REMARKS

Reconsideration of this application is respectfully requested. Consideration and entry of this response is respectfully requested.

REJECTIONS UNDER 35 U.S.C. 103(a)

A. REJECTION OF CLAIMS 1, 2, 4-14 AND 16-17

Claims 1, 2, 4-17 and 20 stand rejected under 35 U.S.C. 103(a) as being unpatentable over previously cited Hurpin (Vaccine, 16: 208-215 (1998)) in view of Hodge (Vaccine, 15: 759-768 (1997), Rice (U.S. Pat. No. 6,127,116) and Lehner (J. Infect. Dis. 179(Suppl 3): S489-92 (1999)). Applicants respectfully traverse this rejection as indicated below.

Independent claim 1, upon which claims 2, 4-17 and 20 depend, is directed to a method for inducing an immune response to a tumor antigen in an animal by administering at least two forms of an antigen into a lymphatic site of an animal in a prime-boost format, where at least one of the forms is administered directly into a lymph node. Applicants maintain that none of Hurpin, Hodge, Rice or Lehner, either alone or when combined with one another, render the instantly claimed methods obvious as discussed below.

Hurpin fails to show administration of two different forms of antigen; animals are administered either an ALVAC or a DNA vector encoding p53. Hurpin is also completely silent as to administration of an antigen directly into a lymph node and administers antigen only via the intravenous, subcutaneous, intradermal, intramuscular, and instrasplenic routes. Direct administration to a lymph node is not mentioned. Finally, Hurpin teaches away from aspects of Applicants' invention that allows for (but do not require) the two different forms of antigen to be administered in different sites (see, for example, p. 211, col. 1, lines 4-5 : ". . . mice primed by the intravenous route and boosted subcutaneously failed to respond.") Applicants maintain that nothing in Hurpin would lead the skilled artisan to be motivated or have any reasonable expectation of success in carrying out the instantly claimed methods. Applicants do not believe Hurpin is a proper 103(a) reference, either alone or in combination with Hodge, Rice and / or Lehner as further discussed below.

Hodge is also silent as to administration of an antigen directly into a lymph node. Hodge administers recombinant avipox and vaccinia vectors encoding human CEA to mice in a prime-boost format. Hodge administers all vectors either intramuscularly or, for prime-boost studies, by tail skin scarification (see p. 761, first column, paragraph entitled "*In vivo* experiments" and p. 762, col. 1 under the heading "Prime and boost studies"). Hodge does not demonstrate, or even mention, administration directly into a lymph node as instantly claimed. Nothing in Hodge would lead the skilled artisan to be motivated to develop or have any reasonable expectation of success in carrying out the instantly claimed methods. Hodge cures none of the deficiencies of Hurpin and Hurpin adds nothing to Hodge. Applicants do not believe Hodge is a proper 103(a) reference, either alone or in combination with Hurpin, Rice and / or Lehner.

To supplement the deficiencies of Hurpin and Hodge, the Examiner turns to Rice and Lehner. Rice does not discuss a protocol involving both a prime and a boost step. As to direct administration to lymph node, Rice's disclosure is limited to the mere mention of this route and nothing more. Indeed, the route is labeled by Rice to be "preferred", but the skilled artisan is provided no further guidance. For example, Rice provides no information as to how to carry out this preferred route. In fact, the route is not mentioned anywhere else in the document. The remainder of Rice's disclosure relates to the cloning of HCV genes. Recognizing these deficiencies in Rice, the Examiner alleges that Rice provides motivation to the skilled artisan to develop the claimed method, and that it is Lehner who provides the reasonable expectation of success. Applicants respectfully disagree.

Lehner teaches only subcutaneous administration of antigen. At p. S489, Lehner states:

> As an alternative strategy, a subcutaneous immunization technique, which aims to administer the vaccine in the proximity of the internal and external iliac lymph nodes, was developed [8]. The same antigen as that administered in the augmented mucosal administration (p27:Ty-VLP) was used, except aluminum hydroxide was used as an adjuvant.

Thus, Lehner states that his technique only "aims to administer vaccine in the proximity" of the lymph node. Aiming to administer an antigen to a lymph node and actually doing so are two different things; Applicants are the only ones to have actually done so.

Lehner's teaching as to direct administration is nothing more than he disclosed nearly five years earlier (Lehner, et al. 1994. J. Immunol. 153: 1858-1868; attached). Thus, over the course of five years, Lehner himself was apparently not motivated to use direct administration.

Lehner provides the skilled artisan with no reasonable expectation of success in directly administering an antigen to a lymph node as part of a prime-boost regimen. At most, Lehner provides an invitation to try direct administration, even when combined with Rice. Applicants maintain that the skilled artisan, having the invitations of Rice and Lehner in hand, would not have had more than a hope that direct administration in a prime-boost format would work at the time of Applicants' invention. It is understood by those of skill in the art that success with advanced techniques such as those instantly claimed is not to be expected until the experiments are completed. Applicants' disclosure represents the first time the skilled artisan could have had a reasonable expectation of success in administering an antigen directly into a lymph node as part of a prime-boost protocol.

Given the cited art's failure to adequately provide the skilled artisan with both the motivation and a reasonable expectation of success in practicing the claimed invention, Applicants respectfully maintain that these rejections are improper. At most, having read Hurpin, Hodge, Rice and Lehner, the skilled artisan may have been curious to try direct administration as part of a prime-boost protocol and hope the procedure would generate an immune response. However, this is not sufficient as hope is not the standard for obviousness. It was not until Applicants actually did the experiments disclosed in their application that the skilled artisan could have known with any reasonable expectation of success that the claimed methods would function as desired. As such, Applicants respectfully request that this rejection be withdrawn.

B. REJECTION OF CLAIMS 18 AND 19

Claims 18 and 19 stand rejected under 35 U.S.C. 103(a) as being unpatentable over previously cited Hurpin (Vaccine, 16: 208-215 (1998)) in view of Hodge (Vaccine, 15: 759-768 (1997), Rice (U.S. Pat. No. 6,127,116), Lehner (J. Infect. Dis. 179(Suppl 3): S489-92 (1999)), Zaremba (Cancer Res. 57: 4750-4577 (1997)) and Salgaller (Cancer

Res. 56: 4749-4757 (1996)). Applicants respectfully traverse these rejections as indicated below.

Claims 18 and 19 are dependent upon claim 17, which is dependent upon claim 1. In part A above, Applicants demonstrated that the combination of Hurpin, Hodge, Rice and Lehner cannot render the inventions of claim 1 obvious. Applicants believe the same reasoning applies to claims 18 and 19 with respect to Hurpin, Hodge, Rice and Lehner. While Zaremba and Salgaller may teach particular sequences relevant to the claims, neither cures the deficiencies of Hurpin, Hodge, Rice and / or Lehner. As such, Applicants believe the combination of Hurpin, Hodge, Rice and Lehner with Zarembe and Salgaller cannot render claims 18 and 19 obvious. As such, Applicants respectfully request that these rejections be withdrawn.

B. REJECTION OF CLAIMS 21-27

Claims 21-27 stand rejected under 35 U.S.C. 103(a) as being unpatentable over previously cited Hurpin (Vaccine, 16: 208-215 (1998)) in view of Hodge (Vaccine, 15: 759-768 (1997), Rice (U.S. Pat. No. 6,127,116), Lehner (J. Infect. Dis. 179(Suppl 3): S489-92 (1999)), and Barnett (Vaccine 15(8): 869-873 (1997)). Applicants respectfully traverse these rejections as indicated below.

Claim 21, upon which claims 22-27 depend, is dependent upon claim 1. In part A above, Applicants demonstrated that the combination of Hurpin, Hodge, Rice and Lehner cannot render the inventions of claim 1 obvious. Applicants believe the same reasoning applies to claims 21-27 with respect to Hurpin, Hodge, Rice and Lehner. The addition of Barnett does nothing to cure the deficiencies of Hurpin, Hodge, Rice and / or Lehner. Barnett may provide the skilled artisan with nucleic acid and protein forms of antigen, but adds nothing to the requirement that at least one of the forms is administered directly to a lymph node. Combination of Rice or Lehner with Barnett provides the skilled artisan with nothing more than an invitation to try direct administration of a nucleic acid and / or protein form of an antigen into a lymph node; this is insufficient support for these rejections. One would not have had a reasonable expectation of success in carrying out the instantly claimed methods until Applicants actually did so, as evidenced by their

application. As such, these rejections are improper and Applicants respectfully request withdrawal of the same.

CONCLUSIONS

Consideration and entry of this amendment is respectfully requested. Applicants respectfully maintain that claims 1, 2 and 4-27 are now in condition for allowance and request that a Notice of Allowance for the pending claims. If the Examiner has any questions or believes a discussion would expedite prosecution, she is encouraged to contact the undersigned as her convenience.

Respectfully submitted,

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Targeted Lymph Node Immunization with Simian Immunodeficiency Virus p27 Antigen to Elicit Genital, Rectal, and Urinary Immune Responses in Nonhuman Primates[1]

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A s.c. route of immunization was developed in non-human primates, which targets the genitourinary-rectal associated lymphoid tissue. A vaccine consisting of rSIV *gag* p27, expressed as hybrid Ty virus-like particles (p27:Ty-VLP) was administered in the proximity of the internal iliac lymph nodes. Secretory IgA and IgG Abs to the p27 Ag were elicited in the vaginal, male urethral, rectal and seminal fluids, urine and serum. Two or more immunodominant B cell epitopes were identified within peptides 51–90 and 121–170 of the sequence of p27, using serum or biliary IgA and IgG Abs. $CD4^+$ T cell proliferative responses to p27 were elicited predominantly in the targeted internal iliac, as well as the inferior mesenteric lymph nodes and the spleen, but not in the unrelated lymph nodes. These cells were then studied for helper function in p27 specific B cell Ab synthesis. Specific IgA and IgG Abs were detected in the same lymphoid tissues as those that displayed proliferative responses. However, cross over reconstitution experiments between splenic and iliac lymph node B and $CD4^+$ T cells suggest that the iliac B cells are essential for specific IgA Ab synthesis, whereas splenic B cells preferentially synthesize IgG Ab. The targeted lymph node (TLN) route of immunization gave comparable B cell, proliferative T cell, and Th cell responses to the vaginal, male genitourinary, and rectal mucosal routes, which were augmented by oral immunization. However, the TLN route induced urinary and seminal fluid sIgA and IgG Abs in addition to genital and rectal Abs. Generating secretory IgA and IgG Abs at the mucosal surfaces, and T and B cell immunity in the regional draining lymph nodes, spleen and circulation by TLN immunization may prevent transmission of virus through the mucosa, dissemination of the virus, and the formation of a latent reservoir of infection. *The Journal of Immunology*, 1994, 153: 1858.

Male and female genital and rectal mucosal transmission of HIV is responsible for a large proportion of patients developing AIDS (1–4). Despite the high prevalence of sexually transmitted diseases the immune system of the female and male genitourinary and rectal tracts have received only limited attention. Secretory IgA and monomeric IgG Abs are found in cervicovaginal and rectal secretions, as well as in the male urethral washings of non-human primates (5–7). IgA-containing plasma cells are found in the lamina propria of these epithelia (8, 9, and L. A. Hussain, L. A. Bergmeier, and T. Lehner; Immunological characterization of male primate urethra. Manuscript in preparation.), and secretory component (SC)[3] necessary for transport of polymeric IgA

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[3] Abbreviations used in this paper: SC, secretory component; Mϕ, macrophages; CTB, cholera toxin B subunit; SIV, simian immunodeficiency virus; TLN, targeted lymph nodes; TT, tetanus toxoid; VLP, virus-like particles; SI, stimulation index.

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across the epithelial cells has been demonstrated in the endocervical (8) and rectal (9) epithelial cells. Cervicovaginal immunization resulted in local sIgA and IgG Abs and in some cases systemic Abs with a variety of Ags (10–14). We were not able to find experimental evidence of male genito-urinary immunization previous to our recent investigation in nonhuman primates (7). Indeed, there appeared to be no preferential homing from the gut-associated lymphoid tissue to the male genital tract of rats (15). Although the rectum has been commonly used for drug administration, there is little information concerning immunization by this route. An early investigation of immunization with poliovirus in the colon elicited local IgA Abs to the virus, which prevented excretion of the virus (16).

Immunization via the mucosal associated lymphoid tissue might be expected to elicit sIgA Abs in the genitourinary and rectal tracts (17, 18). Indeed, oral immunization in rodents induced Abs in the genital tract, and adoptively transferred murine mesenteric lymph node cells home to the genital tissues (17). Direct administration of SRBC into Peyer's patches of Sprague-Dawley rats induced vaginal IgA and IgG Abs (14, 19). These experiments suggest that sensitized B cells may home from gut-associated lymphoid tissue to the genital tract. However, oral immunization in nonhuman primates with the rSIV gag p27 fused to the yeast retrotransposon virus-like particles (p27:Ty VLP) failed to induce significant sIgA Abs in the genital or rectal tract (20). Similarly, microencapsulated formalin-treated SIV administered orally to rhesus macaques failed to elicit vaginal Abs or protection when challenged with live SIV by the vaginal route (21). However, augmenting oral (or intratracheal) immunization by previous i.m. immunization with SIV induced vaginal IgG and IgA Abs to SIV and five out of six macaques were protected when challenged by the vaginal route (21).

Augmenting genital immunization by oral immunization in female (5) or male macaques (7) or augmenting rectal by oral immunization (6) induced sIgA and IgG Abs to p27 at the corresponding mucosal surface. Serum Abs and $CD4^+$ T cell proliferative responses to p27 were also elicited by the three routes of immunization. Autopsy examination of these macaques showed T and B cell responses in the internal iliac lymph node cells, in contrast to systemic immunization. We have administered the hybrid p27:Ty-VLP vaccine mixed with aluminium hydroxide in the proximity of the internal iliac lymph nodes, to target the genitourinary-rectal associated lymphoid tissue, in an attempt to elicit sIgA in the vagina, male urethra, seminal fluid, rectum, and urine of nonhuman primates.

Materials and Methods

Vaccine

The construction of hybrid virus-like particles containing the SIV p27 sequence of isolate 32H of SIVmac251 fused to the p1 protein of Ty has been described previously (22). The SIV *gag* p27 gene was derived from the clone pNIBSCI (23) and the SIV p27:Ty-VLP and control Ty-VLP were purified from yeast extracts. Nonparticulate p27 was prepared by proteolytic cleavage from either p27:Ty-VLP or from a glutathione-*S*-transferase/p27 fusion protein and further purified by ion exchange chromatography (22). The absence of any Ty protein in the p27 preparation was confirmed by Western blotting. The rAgs were covalently linked to cholera toxin B subunit (CTB) (Sigma Fine Chemical Company, Pool, Dorset, UK) at a ratio of 1:1 using (*N*-succinimidyl-3–2 pyridyl dithiopropionate, Sigma Fine Chemical Company) (24).

Immunization schedule

A group of 12 rhesus macaques weighing 4 to 8 kg was used, of which 5 were females and 7 were males. The macaques were housed and maintained according to the guidelines laid down by the United Kingdom Home Office under the Animals Scientific Procedures Act 1986. Of these, 9 macaques were each given 500 μg of p27:Ty-VLP mixed with aluminium hydroxide (AluGel, Uniscience, London, UK) by targeted s.c. injection into the site defined below. Each side was injected with 250 μg of the vaccine in 0.5 ml of sterile saline. Three control animals were similarly immunized with 500 μg of Ty-VLP mixed with aluminium hydroxide. Targeted lymph node (TLN) immunization was conducted twice at monthly intervals. All procedures with the macaques were conducted under sedation with ketamine hydrochloride (10 mg/kg; Southern Veterinary Services, Lewis, Sussex, UK). To compare the s.c. TLN immunization with genito-oral, recto-oral, and i.m. routes of immunization, the corresponding fluids and sera stored from previous experiments were used for quantitative ELISA. Some of the data of the lymphoproliferative assays and in vitro Ab synthesis used for comparison in Figure 2 and Table II have been published separately (5–7).

Collection of fluids

Samples were collected before and at monthly intervals after each immunization. Blood collected from the femoral vessels was defibrinated and the serum separated. A constant volume of about 2.5 ml of vaginal, urethral and rectal washings was collected without trauma, with the aid of flexible, lubricated pediatric nasogastric tubes as described previously (5–7). Seminal fluid was collected by means of electroejaculation (P–T Electronics, Boring, OR 97009) into heparinized bottles. Urine was collected by direct aspiration of the bladder before and approximately 1 mo after each immunization. Bile was aspirated directly from the gall bladder at the necropsy examination.

ELISA

Plates were coated with a predetermined optimal concentration of p27 (at 1 μg/ml) and a random peptide (R20) as a control Ag; they were incubated with doubling dilutions of test samples. Any bound Ab was detected by the second layer rabbit IgG anti-monkey IgA (8 μg/ml; Nordic Immunologic Labs, Maidenhead Berks, UK) or IgG (2 μg; Sigma-Fine Chemicals), followed by affinity-purified goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma-Fine Chemicals) and then *p*-nitrophenyl-phosphate-disodium (Sigma-Diagnostics). The reaction was terminated with 3 M NaOH and the OD was measured at a wavelength of 405 nm. The results were expressed as the lowest dilution giving an OD of 0.15 U above the background sample for all except serum for which an OD of 0.20 U was used. For urethral and rectal washings (concentrated six times), urine (concentrated 10 times), and seminal fluid, the starting samples were used neat and then diluted 1/2, 1/4, etc, whereas serum samples were diluted 1/100, 1/200, etc. The fluids were concentrated by lyophilization and the concentration step for the washings and urine was taken into account when the results were calculated.

Quantitative assays of IgA and IgG Abs to p27

The concentrations of IgA and IgG Abs to p27 were determined by affinity purification of IgA and IgG Abs to p27. Furthermore, to compare IgA Abs to p27 between TLN and four other routes of immunization in the rectal, vaginal, and urethral washings of unknown dilutions, we have prepared total IgA in addition to affinity-purified IgA Abs to p27. Specific IgG and IgA anti-p27 Abs were purified from the serum of an i.m.-immunized macaque by a combination of gel filtration and ion exchange, followed by affinity chromatography. IgG was purified in a single step from DEAE cellulose in 0.01 M Tris phosphate (pH 7.0). IgA was partially purified from the same column in a gradient from 0.01 M phosphate

(pH 7.0) to 0.3 M Tris phosphate (pH 4.0). Fractions containing IgA and some IgG were further purified by negative affinity chromatography, using Sephurose 4B beads (Sigma-Fine Chemicals), coated with anti-human IgG. Both IgG and IgA were then applied to an affinity column of Sepharose beads coated with gag p27 (1 mg/g of beads). Specific IgG and IgA were eluted with 6 M guanidine hydrochloride brought to neutrality and dialyzed against PBS. The concentrations of IgG and IgA were determined by spectrophotometry (at E_{280} nm). The concentration of specific IgG and IgA Abs to p27 were expressed by using the corresponding affinity-purified IgG and IgA p27 Abs as standards in each assay. In addition, the concentrations of IgA Abs to p27 were expressed as a percentage of total IgA concentration in the test fluid.

Characterization of urethral fluid, urine, and seminal fluid for IgA Abs

These were examined by ELISA for IgA and IgG Abs and for the presence of secretory component and J chains (5), by means of goat anti-human secretory component Abs (Sigma), and goat anti J-chain Abs (Nordic).

Mapping of B cell epitopes by competitive inhibition assays

Serum and biliary IgA and IgG Abs were determined to 22 overlapping peptides which were derived from the sequence of p27. These peptides were 20 mers overlapping by 10 residues and were supplied by Dr H. Holmes (Repository of the Medical Research Council's AIDS Directed Programme, ADP 714; peptides 1–22). Peptides to which raised Ab levels were found in the immune sera and in specimens of bile were selected for competitive inhibition assays. Plates were coated with p27 (1 μg/ml) as described for ELISA, and 0, 25, 50, and 100 μg/ml of each of the 10 preselected peptides, a random peptide (R20) or p27 was added to each well and then incubated with doubling dilutions of the test serum or bile. The Ab binding ELISA results were expressed as the percentage inhibition by each peptide of Ab binding to the p27 Ag.

Lymphoproliferative assay

Mononuclear cells were separated from defibrinated blood by Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation, as described previously (5). The spleen, internal iliac, inferior mesenteric, iliac, para-aortic, superior mesenteric, bronchial, and axillary lymph nodes were removed at autopsy from six immunized macaques. The cells were separated after breaking up the tissues and processed. They were then cultured without Ag and with 1 and 10 μg/ml of either p27, p27:Ty-VLP, Ty-VLP, R20, or Con A in 96-well round-bottom plates (Costar, Cambridge, MA) containing RPMI 1640 (Sigma), supplemented with 100 μg/ml penicillin (Sigma) and 100 μg/ml streptomycin, 2 mMol/l L-glutamine (Sigma) and 10% autologous heat-inactivated serum for 4 days and then pulsed with 0.5 μCi [^{3}H]thymidine for 4 h. The cells were then harvested onto filter paper discs and the [^{3}H]thymidine uptake was determined by scintillation counting. The results were expressed as stimulation indices (ratio of counts with and without Ag), and as cpm for cultures stimulated with 10 μg/ml of p27; those stimulated with p27:Ty-VLP gave similar results (data not shown). All cultures yielded high stimulation indices and counts with Con A, and no significant increase in counts was seen with R20 (data not presented).

To determine whether the CD4 or CD8 T cell subset responds to the p27 Ag, the mononuclear cells were incubated on plastic plates in RPMI 1640 (containing 10% FCS) for 1 h at 37°C in a humidified atmosphere with 5% CO_2. Nonadherent cells were removed and adherent cells (enriched for monocytes) were incubated with RPMI 1640 containing 10% FCS overnight at 37°C and recovered by washing the plate. The nonadherent T cells were then separated by rosetting with 2-aminoethylisothiouronium bromide-treated SRBC. The rosetting T cells were further separated by panning 5×10^6 cells with a predetermined optimal amount of anti-T4 mAb (100 μl culture supernatant per 10^6 cells) in HBSS with 10% FCS overnight at 4°C. After washing, 1.5×10^7 cells were added to petri dishes that had been coated with affinity purified goat anti-mouse IgG (at 5 μg/ml in 0.5 M Tris-HCL pH 9.5) for 70 min at 4°C. The nonadherent cells consisted of enriched $CD8^+$ cells and the adherent cells were enriched $CD4^+$ cells. The enriched T cell subsets were reconstituted with 10% monocytes (plastic adherent cells) and stimulated with p27 and the other Ags as described above.

In vitro $CD4^+$ Th cell function in B cell Ab synthesis

IgA and IgG Ab synthesis was studied with enriched B cells, CD4 cells, and monocytes derived from the spleen or internal iliac lymph nodes. Enriched B cells (10^5), $CD4^+$ cells (4×10^5) and monocytes (5×10^4) were reconstituted and stimulated with p27, Ty-VLP or tetanus toxoid (TT) (200 or 20 ng/ml) for 7 days, washed, and then cultured without Ag for 8 days (5, 25). The culture supernatants were assayed for the corresponding IgA or IgG Abs by a modified ELISA. Microtiter plates were coated with 1 μg/ml of Ag (p27, Ty-VLP, or TT) and culture supernatants were added at a dilution of 1/2. Bound Ab was detected using goat anti-monkey IgA or IgG (Nordic), followed by biotinylated rabbit anti-goat IgG Ab, horseradish peroxidase, and phenylenediamine dihydrochloride. The reaction was terminated with 2 M H_2SO_4. The results are expressed as OD at a wavelength of 492 nm, with the OD of the control culture (without Ag) subtracted.

Cross-over reconstitution experiments between iliac lymph node and splenic cells

Cross-over reconstitution experiments were then conducted to determine if the $CD4^+$ Th and B cell Ab functions differ between the splenic and internal iliac lymph node cells in IgA and IgG Ab synthesis. Enriched $CD4^+$ T cells and B cells were reconstituted as described above, followed by mixing splenic $CD4^+$ cells with iliac lymph node B cells and Mϕ), and vice versa. A comparison was then made between the splenic and iliac lymph node $CD4^+$ T cells and B cells after immunization by the s.c. TLN or i.m. route.

Statistical analysis

The results were analyzed by applying the paired or unpaired Student t-test.

Results

Genitourinary-rectal-associated lymphoid tissue

Autopsy examination of rhesus and cynomolgus macaques after mucosal genital or rectal immunization revealed enlarged internal and external iliac lymph nodes containing activated T and B cells sensitized to the immunizing Ag (5–7). The internal iliac lymph nodes were also identified after vaginal submucosal administration of colloidal iron in rhesus macaques (26) and are consistent with the lymph nodes identified by subserous immunization of the female mouse pelvis (13). The localization of the iliac lymph nodes was studied in five autopsies by s.c. injection of 0.5 ml of India ink, aimed to be deposited in the proximity of the internal iliac lymph nodes before the autopsy. The internal iliac lymph nodes were found distal to the femoral vessels and about 2 to 4 cm s.c.

IgA and IgG Abs in vaginal, male urethral and rectal washings, urine, seminal fluid, and serum

Sequential IgA Abs to p27 were studied in six macaques and these showed after the first TLN immunization a significant increase in Abs in vaginal washings of female ($p < 0.05$) or urethral washings of male macaques ($p < 0.01$) and in rectal washings of either sex ($p < 0.001$) (Table I). Increases in Abs were also detected after the second immunization; vaginal IgA Abs increased approximately threefold, rectal and urethral IgA Abs twofold. A

Table I. *IgA and IgG Abs to p27 after targeted lymph node immunization (× 2) with p27:Ty-VLP in AluCel; No. 1 to 3 female and 4 to 6 male macaques*[a]

		Abs[a]						
		Immunization[b]		Immunization[b]		Immunization		
No.	Ig	Post-1	Post-2	Post-1	Post-2	Pre-	Post-1	Post-2
		Vaginal Fluid*		Rectal Fluid*			Serum**	
1	IgA	250	822	55	200	6.0	10.7	141.4
2	IgA	328	1098	58	137	27.0	59.3	162.8
3	IgA	496	1172	81	109	21.5	33.4	59.6
Mean		360.7	1030.7	64.7	106.7	18.2	34.5	121.3
(± SEM)		(70.6)	(106.5)	(8.2)	(31.9)	(6.3)	(14.0)	(31.5)
1	IgG	56	92	14.0	34.0	4.9	37.2	111.3
2	IgG	35	150	14.0	38.0	4.0	73.6	97.9
3	IgG	88	92	20.0	23.0	21.3	65.7	69.8
Mean		59.7	111.3	16.0	31.7	10.1	59.0	93.0
(± SEM		(15.4)	(19.3)	(2.0)	(4.5)	(5.6)	(10.9)	(12.2)
		Urethral Fluid*		Rectal Fluid*			Serum**	
4	IgA	66	140	44	69	14.4	39.1	59.5
5	IgA	58	138	52	104	8.6	14.4	54.9
6	IgA	70	144	24	33	ND	101.7	136.8
Mean		64.7	140.7	40.0	68.7	11.5	51.7	83.7
(± SEM)		(3.5)	(1.7)	(8.3)	(20.5)		(25.9)	(26.6)
4	IgG	65	55	13.0	19.0	14.5	78.0	60.4
5	IgG	14	23	17.0	20.	9.0	24.4	62.2
6	IgG	14	27	7.0	10	-	108.9	109.7
Mean		31.0	35.0	12.3	16.3	11.8	70.4	77.5
(± SEM)		(17.0)	(10.1)	(2.9)	(3.2)		(24.7)	(16.2)

[a] Expressed in nanograms per milliliter (*) or ** micrograms per milliliter (**) before and mo after the first and second immunization.
[b] Preimmunization IgA or IgG Abs to p27 were not detected in vaginal, urethral, or rectal fluids. Three Ty-VLP immunized macaques did not show significant p27-specific IgA or IgG Abs.

significant increase in serum IgA Abs to p27 was found in all macaques ($p < 0.05$). None of the three control macaques immunized with Ty-VLP yielded p27 Abs, but Abs to Ty were detected (results not presented). Examination for IgG Abs showed increases in p27 Abs in vaginal, urethral, and rectal washings, which, however, failed to reach the 5% level of significance (Table I). Serum IgG Abs were significantly increased in all macaques ($p < 0.02$).

In view of the unknown dilution factor in collecting mucosal washings, the results were then expressed as a percentage of p27 Abs within the total IgA concentration of that fluid (Table II). The highest proportion of IgA Abs to p27 was found in serum of female (3.97% ± 0.48) or male (3.33 ± 0.54) macaques, vaginal washings (3.79% ± 0.38), and seminal fluid (3.3% ± 0.15); this decreased progressively in rectal washings (1.7% ± 0.24), urine (0.78% ± 0.18) and urethral washings (0.6% ± 0.12). Urine and seminal fluid were also collected from three controls immunized with Ty-VLPs and IgA or IgG Abs to p27 were not detected. However, all three macaques elicited significant serum IgA and IgG Abs to Ty.

Comparative quantitative analysis of p27-specific IgA Abs after five routes of immunization

The highest proportion of p27-specific IgA Abs was found in the sera of male and female macaques immunized by the TLN route (3.3 to 3.9%), and these were significantly greater ($p < 0.05$) than those after the recto-oral, male genito-oral, or i.m. route of immunization (1.9 to 2.8%; Table II). Rectal washings showed similar proportions of p27 specific IgA Abs after TLN (1.7% ± 0.24) as those after mucosal immunizations (1.6 to 1.7%), with the exception of recto-oral immunization (1.2% ± 0.26). The proportion of vaginal p27-specific IgA Abs was significantly higher ($p < 0.02$) after TLN (3.8% ± 0.38) than that after vagino-oral immunization (2.3% ± 0.28). Similarly, seminal fluid p27-specific IgA Abs were higher after TLN (3.3% ± 0.15) than male genito-oral immunization (1.4%), but the number of samples was inadequate for statistical analysis. Urinary and urethral p27-IgA Abs were comparable with those after male genito-oral immunization. Intramuscular immunization failed to elicit any mucosal, urinary, or seminal fluid p27-specific IgA Abs (Table II).

Table II. *Comparative quantitative analysis of SIV anti-p27 IgA Abs, after five routes of immunization in 29 macaques with SIV p27:Ty-VLP*[a]

Route of Immunization	No.	IgA Abs to p27[b]					
		Rectal	Vaginal	Urethral	Seminal fluid	Urine	Serum
1a TLN (male)	5	1.70 (0.41)		0.61 (0.12)	3.3 ± 0.15	0.78 (0.18)	3.33 (0.54)[c]
1b TLN (female)	4	1.83 (0.27)	3.79 (0.38)[d]			0.77 (0.12)[e]	3.97 (0.48)[c]
2 Vagino-oral-IM	5	1.70 (0.36)	2.27 (0.28)	0	0		3.03 (0.14)
3 Genito-oral-IM (male)	4	1.60 (0.05)		0.89 (0.23)	1.4[f]	0.84 (0.11)	1.96 (0.05)
4 Recto-oral-IM (male)	6	1.16 (0.26)		0		0	2.81 (0.12)
5 Intramuscular	5	0	0	0		0	2.34 (0.29)

[a] Data for groups 2 to 5 were obtained by ELISA of fluids collected in previous experiments.
[b] The concentration of p27 IgA Abs is expressed as percentage of total IgA concentration and is given as mean % (± SEM).
[c] $p < 0.05$ of serum IgA: 1a against 3, 1a and 1b against 4 or 5.
[d] $p < 0.02$ of vaginal washings: 1b against 2.
[e] $n = 3$.
[f] $n = 2$.

Table III. *Competitive inhibition of serum or biliary IgA and IgG binding to recombinant p27 Ag by increasing concentrations of 10 selected peptides derived from the sequence of p27; a random synthetic 20[mer] peptide (R20) was used as a negative control and the p27 Ag as a positive control*[a]

Peptide	IgA						IgG					
	Serum			Biliary			Serum			Biliary		
	25[b]	50	100	25	50	100	25	50	100	25	50	100
R20	0	10	10	1	14	6	12	13	15	8	15	15
p27	52	82	100	52	65	86	48	71	82	52	71	86
21–40	26	32	33	35	24	23	30	34	34	10	23	27
31–50	24	17	17	5	26	23	40	42	48	12	26	26
41–60	25	25	17	25	36	34	36	36	43	28	35	34
51–70	45	50	51	27	27	30	60	66	74	28	35	35
61–80	35	58	73	48	69	79	62	69	82	59	68	82
71–90	41	65	82	57	72	92	56	79	94	60	74	88
121–140	37	76	86	47	79	92	65	67	76	48	82	92
131–150	40	68	65	60	60	69	59	70	84	63	75	79
141–160	34	73	89	48	70	90	54	67	86	45	72	90
151–170	39	56	62	39	60	66	64	70	80	40	62	55

[a] The serum and bile were from a TLN-immunized macaque.
[b] Increasing concentrations of 25, 50, and 100 μg/ml of peptides were used for inhibition. Inhibition greater than 50% is set in bold face type.

Characterization of vaginal, urethral, rectal, urinary, and seminal fluid IgA Abs

All fluids were examined by ELISA for secretory component and J chains in IgA. Abs to p27 in these fluids were detected by IgA, secretory component or J chain Abs. The p27-specific Ab titers were comparable for IgA and SC (1/4 to 1/8), and to a lesser extent for J chains (1/2 to 1/8). In contrast, IgA anti-p27 Abs in serum failed to show detectable secretory component or J chains. These results are consistent with the p27-specific IgA Abs in vaginal, seminal, urethral, rectal fluids, and urine, being polymeric secretory IgA, whereas serum IgA is predominantly monomeric.

Mapping of B cell epitopes by competitive inhibition assays

Competitive inhibition with increasing concentrations of p27 peptides showed significant inhibition (>50%) with serum and biliary IgA and IgG Abs in the TLN immunized macaques by two groups of polypeptides 61–90 and 121–170 (Table III). Maximum inhibition within these two groups varied with different peptides. Peptide 51–70 showed significant inhibition with serum IgG and possibly IgA Abs. The unrelated random peptide (R20) showed little inhibition, as did three other p27-derived peptides (21–60), whereas p27 inhibited Abs to p27 by 79 to 100% (Table III). There was no obvious difference in the B cell epitopes between the serum and biliary Abs, except for residues 51–70 that significantly inhibited serum but not biliary IgA and IgG Abs to p27 (Table III). These results suggest that two or more immunodominant B cell epitopes were elicited by the TLN route within the polypeptides 61–90 and 121–170, but the fine specificities of the B cell epitopes within these residues have not been determined.

Table IV. *Comparative inhibition assays of biliary IgA (1a-5a) and IgG (1b-5b) Abs to p27 by synthetic peptides derived from the sequence of p27*[a]

Route of Immunization	Sex	Percent Inhibition with 100 μg/ml Peptides					
		R20	p27	21–40 31–50 41–60	51–70	61–80 71–90	121–140 131–150 141–160 151–170
Biliary IgA							
1a TLN	Male	6	86	23–23–34	30	79–92	92–69–90–66
1a TLN	Female	7	82	19–15–30	29	74–91	92–73–91–51
2a Genito-oral	Female	6	82	34–6–36	10	49–81	82–52–82–57
3a Genito-oral	Male	28	100	24–18–20	36	74–68	42–71–78–69
4a Recto-oral	Male	4	100	26–16–10	51	69–78	62–59–92–63
5a Intramuscular		20	79	7–6–4	61	74–88	57–42–72–60
Biliary IgG							
1b TLN	Male	15	86	27–26–34	35	82–88	92–79–90–55
1b TLN	Female	22	86	23–14–43	29	78–92	90–76–90–54
2b Genito-oral	Female	29	90	31–29–31	31	63–57	61–50–56–54
3b Genito-oral	Male	27	100	31–8–38	36	84–77	65–72–84–68
4b Recto-oral	Male	26	80	0–0–0	60	71–87	63–78–75–70
5b Intramuscular		33	100	31–24–23	67	80–80	73–46–82–64

[a] Adjacent peptides yielding similar inhibition were grouped together; polypeptides (21–60), (61–90) and (121–70).
Three concentrations (25, 50, and 100 μg/ml) of each peptide (or p27) were used in the inhibition assays (as in Table III) but only maximal inhibition with 100 μg/ml of peptide is given. Inhibition greater than 50% is set in bold face type.

Comparative inhibition assays

The competitive peptide inhibition assay was used to compare B cell epitopes in biliary IgA and IgG Abs, following the five routes of immunization (Table IV). Bile was used for the comparative inhibition assays because human bile contains both polymeric (54.1 μg/ml) and monomeric (23.0 μg/ml) IgA, in addition to IgG (88.8 μg/ml) (27), so that macaque bile is likely to assay both types of IgA. As with TLN immunization B cell epitopes were found for IgA and IgG Abs within residues 61–90 and 121–170 following the other routes of immunization. Only peptide (51–70) inhibited significantly biliary IgA and IgG Abs after i.m. or recto-oral immunization, but not after TLN or genito-oral immunization (Table IV). No significant inhibition was detected within the polypeptide 21–60 or the random peptide R20, but 79 to 100% inhibition was found with the recombinant p27 Ag (Table IV). The results suggest that B cell epitopes reside within the polypeptides 61–90 and 121–170, irrespective of the route of immunization, but that a flanking peptide 51–70 can be found in macaques immunized by the i.m. or recto-oral route.

Lymphoproliferative responses in PBMC, spleen, and lymph node cells

T cell proliferative responses of PBMC to p27 (or Ty) showed a mean SI of 1.8 ± 0.31 before immunization, which increased significantly after the first (mean SI 5.9 ± 1.9) and especially after the second TLN immunization (mean SI 18.6 ± 5.3; Fig. 1). This was specific to p27 (and Ty, not presented), but there was no change with the random peptide or Con A stimulation (the latter induced SI from 27.0 to 347.0 in all assays). The three control macaques immunized with Ty-VLP did not respond to stimulation with p27, but they responded to stimulation with Ty (SI, 3.4, 6.0, and 2.8). T cells sensitized to p27 (and Ty) were found at autopsy in the spleen, internal and external iliac, inferior mesenteric, and to a lesser extent, iliac-paraortic lymph nodes, but not in the superior mesenteric, bronchial, or axillary lymph nodes (Fig. 2). The proliferating T cells belonged to the CD4 subset, as enriched $CD4^+$ T cells from blood, spleen, iliac, or inferior mesenteric lymph nodes showed SI of 3.2 to 6.0, whereas enriched $CD8^+$ T cells failed to respond to p27 (SI, 0.7 to 1.9; data not presented).

Comparative proliferative responses of T lymphocytes between TLN and four routes of immunization

TLN immunization elicited a similar pattern of responses to p27:Ty-VLP in the lymph nodes as the three mucosal routes; internal iliac, inferior mesenteric, and iliac-paraortic, in addition to the splenic and circulating T lymphocytes (Fig. 2). These four routes of immunization differed from the i.m. route in which only the splenic and circulating T cells and none of the lymph node cells responded to p27. The magnitude of T cell proliferation after TLN immunization was similar to that found after genito-oral immunization in females, but lower than those after genito- or recto-oral immunization in males (Fig. 2).

Assay of splenic and iliac lymph node $CD4^+$ Th cell function in B cell Ab synthesis

To find out if TLN immunization can elicit splenic or lymph node $CD4^+$ T cell help for B cell Ab synthesis,

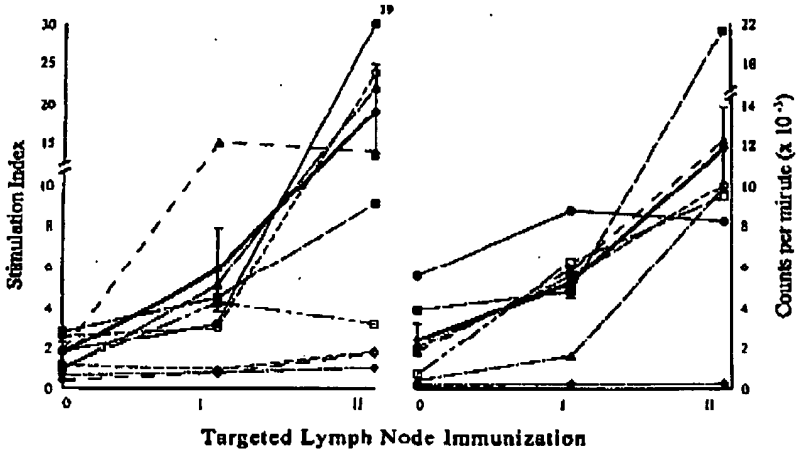

FIGURE 1. Lymphoproliferative responses to SIV p27 by PBMC in six macaques before and 1 mo after each of the two targeted lymph node immunizations with p27:Ty-VLP in aluminium hydroxide. Each macaque is given a different symbol and the mean ± SEM (n = 6) is shown by the bold line. The three control macaques immunized with Ty-VLP in aluminium hydroxide did not respond to p27 (bottom of the graph), but showed significant stimulation indices with Ty-VLP.

these cells were reconstituted in vitro with Mϕ and stimulated with p27. Reconstituted splenic cells showed both IgA and IgG Abs to p27, but the IgG titers were higher than those for IgA (Table V). The internal iliac lymph node cells also yielded IgA and IgG Abs to p27 but, with one exception, the IgA Abs were similar to IgG Abs (Table V). Reconstituted inferior mesenteric lymph node cells showed similar IgA and IgG Ab titers to p27 (data not presented). These results suggest that TLN immunization elicits IgA and IgG Abs to the stimulating Ag, but whereas the lymph node lymphocytes yield comparable IgA and IgG Ab levels, the splenic cells showed higher IgG than IgA Abs.

Comparative CD4⁺ Th cell function in B cell Ab synthesis between TLN and four routes of immunization

Reconstituted splenic CD4⁺ T cells, B cells, and Mϕ showed consistently higher p27-specific IgG than IgA Ab synthesis after all but the recto-oral route of immunization where the levels were equally low (Table VI). In contrast, reconstituted iliac lymph node cells yielded comparable p27-specific IgA and IgG Abs after TLN, higher IgA than IgG Abs after any of the three mucosal immunizations and no Abs after the i.m. route of immunization (Table VI).

Cross-over reconstitution experiments between iliac lymph node and splenic cells

The preferential synthesis of p27-specific IgG by reconstituted splenic CD4⁺ T cells, B cells, and Mϕ and comparable IgA and IgG Abs by iliac lymph node cells after TLN immunization were further examined by cross-over reconstitution experiments (Table VII). Reconstitution of CD4⁺ splenic with B iliac (instead of splenic) cells and Mϕ showed a decrease in IgG Abs in the three experiments but an increase in IgA Abs in two out of the three experiments (Table VII(a) and (b)). Conversely, reconstitution of CD4⁺ iliac with B splenic (instead of iliac) cells and Mϕ resulted in a loss or decrease in p27-specific IgA Abs, without a change in IgG Abs in two of the three experiments (Tables VII(c) and (d)). Thus, optimum IgA Ab synthesis to p27 requires iliac B cells and either iliac or spleen CD4⁺ cells, whereas IgG Abs can be generated by both splenic and iliac CD4⁺ T cells and B cells, although maximum levels of IgG Abs are elicited by reconstitution with splenic CD4 and B cells. To find out if the isotype preferences of splenic and iliac lymph node cells is specific to TLN immunization, we conducted similar cross-over reconstitution studies after i.m. immunization. These showed that unlike splenic cells, the iliac lymph node cells failed to generate IgA or IgG Abs (Table VII(a) and (c), experiments 4 and 5). However, reconstitution of CD4⁺ iliac with splenic B cells elicited significant IgG Abs in both IgA and Abs in one experiment (Table VII(d), experiments 4 and 5), but splenic CD4⁺ cells reconstituted with iliac B cells resulted in loss of both IgG and IgA Abs (Table VII(b), experiments 4 and 5).

Discussion

Targeting a recombinant vaccine in the proximity of the principle genitourinary and rectal draining lymph nodes in nonhuman primates elicited four levels of immunity: mucosal, draining lymph nodes, the spleen, and circulation. Specific secretory IgA and IgG Abs have been induced in vaginal, male urethral, rectal and seminal fluids, and in urine. The sIgA Abs may act as a barrier of viral adhesion to the epithelial surface of the genital, urinary, or rectal tract. The presence of specific sIgA (and IgG) Abs in urine may help urethral Abs to prevent adhesion of SIV to the mucosa, and from infecting the urinary tract. The function of IgA and IgG Abs in seminal fluid might prevent or

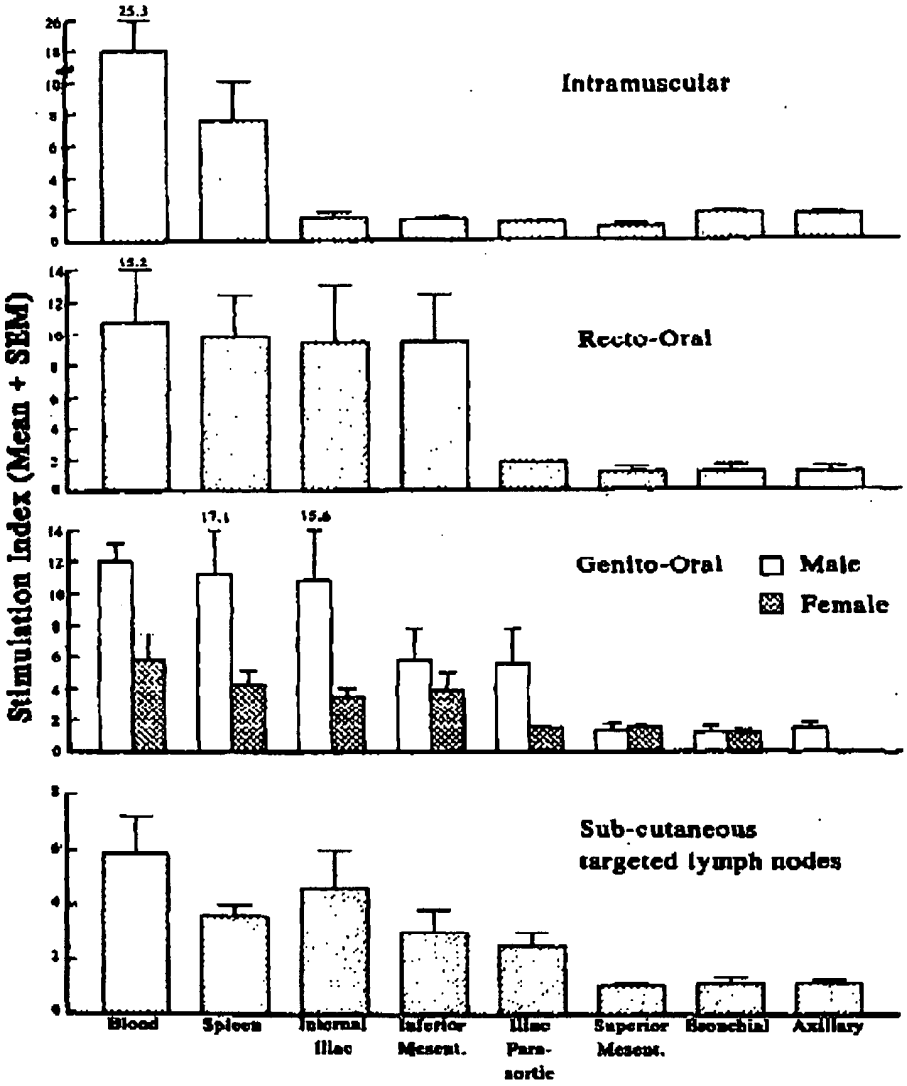

FIGURE 2. Comparative proliferative responses of T lymphocytes from 19 rhesus macaques immunized with p27:Ty-VLP mixed with aluminum hydroxide by the TLN route (n = 6), and i.m. route (n = 3), or with p27:Ty-VLP linked to CTB subunit, administered by the recto-oral (n = 3), male genito-oral (n = 4), or female genito-oral route (n = 3).

diminish transmission of HIV to the sexual partner, possibly by the Abs masking HIV antigenic binding sites to the epithelium, thereby causing viral exclusion. However, sIgA may also prevent viral assembly inside epithelial cells which express the polymeric receptors for IgA (28). A potential neutralizing function of IgG Abs at the mucosal surface has so far not been investigated.

As mucosal immune responses can be achieved by the TLN route of immunization, it seemed important to compare the immune responses with those elicited by direct vaginal, male genitourinary, or rectal mucosal immunization. Examination of the proportion of IgA Abs to p27 in each fluid after TLN immunization with the rSIV p27:Ty-VLP and aluminium hydroxide suggests the following. (1) Genital, urinary, rectal, seminal fluid, and serum IgA and IgG Abs were elicited. (2) Vaginal, urethral, rectal fluid, and urinary IgA Abs to p27 are polymeric (with J chains) and secretory (with SC). (3) Higher concentrations of vaginal, rectal, urinary, and serum IgA Abs are achieved after TLN than after vagino-oral immunization. (4) IgA Ab concentrations in serum were higher, in rectal fluid and urine they were similar, and in seminal and urethral fluid they

Table V. *IgA and IgG Ab synthesis by stimulation in vitro with p27, Ty-vLP, or TT of reconstituted CD4⁺ cells, B cells and macrophages from the spleen and iliac lymph nodes of six macaques immunized with p27:Ty-VLP by the TLN route*[a]

Expt. No.	Ag	Spleen		Iliac	
		IgA	IgG	IgA	IgG
1	p27	0.55 (1:4)	0.97 (1:8)	ND	ND
	Ty-VLP	0.11	0.13	ND	ND
	TT	0.05	0.04	ND	ND
2	p27	0.56 (1:2)	1.7 (1:8)	0.99 (1:4)	0.98 (1:16)
	Ty-VLP	0.02	0.01	0.14	0.16
	TT	0.0	0.01	0	0
3	p27	0.16 (1:1)	0.46 (1:4)	0.52 (1:2)	0.25 (1:2)
	Ty-VLP	0.06	0.04	0.07	0.02
	TT	0.04	0.05	0.03	0.05
4	p27	0.21 (1:1)	1.6 (1:16)	0.50 (1:2)	1.4 (1:8)
	Ty-VLP	0.12	0.13	0.10	0.11
	TT	0.06	0.10	0.0	0.0
5	p27	0.44 (1:2)	0.62 (1:4)	0.53 (1:4)	0.48 (1:2)
	Ty-VLP	0.12	0.04	0.03	0.06
	TT	0.02	0.04	0.04	0.01
6	p27	0.12 (0)	0.23 (1:2)	0.18 (1:1)	0.16 (1:1)
	Ty-VLP	0	0	0	0
	TT	0.08	0.08	0.07	0.05

[a] Abs to p27 were estimated by ELISA and the results are expressed as absorbance at a wavelength of 492 nm and as a dilution (titer) of the cell supernatant in parentheses.

Table VI. *Comparative in vitro Ab synthesis by reconstituted CD4⁺ T cells, B cells, and macrophages with p27 from macaques immunized by five routes with p27:Ty-VLP mixed with aluminium hydroxide ($Al(OH)_3$), (routes 1a, 1b, and 5), or linked to CTB (routes 2 to 4)*[a]

Route of Immunization	No. of Macaques	Adjuvant	Iliac Cells		Splenic Cells	
			IgA	IgG	IgA	IgG
1a TLN (male)	3	$Al(OH)_3$	**0.40** (0.11)	**0.68** (0.37)	**0.26** (0.10)	**0.82** (0.40)
1b TLN (female)	3	$Al(OH)_3$	**0.76**[b]	**0.62**[b]	**0.42** (0.13)	**1.04** (0.36)
2 Genito-oral (female)	3	CTB	**0.30** (0.03)	**0.19** (0)	**0.20** (0.02)	**0.40** (0)
3 Genito-oral (male)	3	CTB	**0.56** (0.07)	**0.49** (0.06)	0.12 (0.05)	**0.24** (0.07)
4 Recto-oral (male)	3	CTB	**0.20** (0.05)	0.04 (0)	**0.21** (0.04)	**0.19** (0.01)
5 Intramuscular	3	$Al(OH)_3$	0.05 (0)	0.08 (0)	**0.27** (0.12)	**0.50** (0.25)

[a] The results of p27 Abs are given as mean (± SEM) of absorbance at 492 nm.
[b] Two macaques tested only.

were slightly lower after TLN than those found after male genito-oral immunization. (5) Serum and rectal IgA Abs to p27 showed higher concentrations after TLN than recto-oral immunization, and only the former elicited detectable Abs in the urine and urethral washings.

The immunodominant B cell epitopes were then determined after TLN immunization by using overlapping synthetic peptides derived from the sequence of p27. A competitive inhibition assay was used in which the peptides were competing for Ab binding with the recombinant p27 protein. Inhibition of 55 to 92% (mean 78%) was recorded with polypeptides 121–170 and 73–94% (mean 84%) with polypeptides 61–90, but less than 35% inhibition (mean 25%) was detected with polypeptides 21–60 and 6 to 15% with the random peptide R20. These results suggest that two or more B cell epitopes reside within polypeptides 61–90 and 121–170. A comparative inhibition assay with biliary IgA or IgG Abs in macaques immunized by the i.m., recto-oral, or genito-oral route confirmed these B cell epitopes. However, peptide 51–70, flanking the polypeptide 61–90, showed significant inhibition of biliary IgA or IgG Abs only from those macaques immunized by the i.m. and recto-oral routes, and might be part of one or more B cell epitopes within residues 51–90. The minimal peptide capable of binding IgA or IgG Abs will have to be determined, although there are inherent difficulties in using linear synthetic peptides which do not have the conformational structure of the native protein. Nevertheless, the B

Table VII. *The effect of cross-over reconstitution of splenic (spl) with iliac lymph node CD4 and B cells on in vitro IgA and IgG Ab synthesis to p27 after TLN or i.m. immunization*[a]

Expt. No.	Route of Immunization	Reconstitution of Cells: (a) CD4 spl + B spl IgA	(a) CD4 spl + B spl IgG	(b) CD4 spl + B iliac IgA	(b) CD4 spl + B iliac IgG	(c) CD4 iliac + B iliac IgA	(c) CD4 iliac + B iliac IgG	(d) CD4 iliac + B spl IgA	(d) CD4 iliac + B spl IgG
1	TLN	**0.16**	**0.46**	**0.52**	**0.24**	**0.52**	**0.25**	0.15	**0.28**
2	TLN	**0.21**	**1.63**	**0.39**	**0.59**	**0.49**	**1.37**	0.14	**0.34**
3	TLN	**0.56**	**1.69**	**0.26**	**0.36**	**0.99**	**0.98**	**0.78**	**1.08**
4	Intramuscular	**0.16**	**0.36**	0.10	0.12	0.14	0.15	0.13	**0.23**
5	Intramuscular	**0.52**	**1.04**	0.09	0.05	0.01	0.03	**0.29**	**0.84**

[a] Macrophages were added to all reconstitution experiments; the results are given as absorbance at 492 nm and those greater than 0.15 are set in bold face type.

cell epitopes reside within peptides that overlap or are adjacent to the T cell epitopes 121–150, 41–60, and 61–80, mapped for T cells after oro-rectal and i.m. immunization (6, and R. Brookes, L. A. Bergmeier, S. E. Adams, and T. Lehner. 1994. Generation of diversity in T cell epitope hierarchy by different routes of immunization with SIV p27:Ty-virus like particles. Manuscript in preparation.).

A comparison of the lymphoproliferative responses after TLN with those of the three mucosal routes of immunization showed no significant difference in the distribution of lymph node cells sensitized to the p27 Ag. The proliferative T cells belonged to the $CD4^+$ subset, irrespective of the route of immunization (5, 6, 7). It is interesting that the magnitude of T cell proliferation after TLN immunization was similar to that found after female genito-oral but lower than those after male genito-oral or recto-oral immunization. TLN, like the mucosal routes differed from IM immunization by sensitization of $CD4^+$ T cells not only in the circulation and the spleen but also in the internal iliac, inferior mesenteric, and common iliac-para-aortic lymph nodes.

Localization of specific T and B cells to the primary draining lymph nodes of the genitourinary and rectal tracts might be essential in preventing transmission of HIV by infected Langerhans, dendritic cells, or Mϕ from the mucosal tissues to these lymph nodes. The T and B cell functions might prevent viral latency and producing a viral reservoir in the primary draining lymph nodes, if the mucosal immune barrier were to be breached. The significance of this observation has been greatly enhanced recently by the evidence that HIV is found early in infection in the lymph nodes, and that the virus remains latent probably in the follicular dendritic cells (29, 30). A central immune barrier is also induced, which is comparable to that found after i.m. immunization, in that splenic and circulating proliferative and Th cells, as well as sensitized B cells, IgG and IgA Abs, are found.

Reconstitution experiments with enriched $CD4^+$ T cells, B cells, and Mϕ separated from the spleen and iliac lymph nodes after TLN immunization elicited SIV p27 specific IgA and IgG Abs. However, although splenic cells elicited higher IgG than IgA Abs, the iliac lymph node cells yielded comparable IgG and IgA Abs. A comparison of TLN with the three mucosal and the i.m. routes of immunization showed similar results with reconstituted splenic cells, in that p27-specific IgG Abs were higher than the IgA Abs. However, reconstituted iliac lymph node cells yielded higher IgA than IgG Abs after the three mucosal routes of immunization, similar IgA and IgG Abs after TLN, and no Abs after i.m. immunization. It appears that immunization by the TLN route elicits regional and central $CD4^+$ Th cells and B cells, as was found in genito-oral or recto-oral immunization, but the helper function was higher especially for IgG Abs. Surprisingly, p27-specific IgG Ab synthesis by splenic cells was higher than those found after i.m. immunization. Cross-over reconstitution experiments between iliac and splenic $CD4^+$ T and B cells and Mϕs were consistent with the concept that higher IgG Ab synthesis is generated by splenic than iliac B cells, and conversely higher IgA Abs are synthesized by B cells from the iliac lymph nodes than those from the spleen. $CD4^+$ Th cells were essential for both IgG and IgA synthesis in this reconstitution system (31). These results are consistent with the murine studies that oral mucosal immunization induces predominantly IgA Abs, unlike i.m. immunization, which favors IgG Abs (32–35). It is noteworthy that i.m. immunization elicited maximal IgG and IgA Ab responses by splenic CD4 and B cells and failed to elicit significant IgA or IgG Abs from the iliac lymph node cells.

TLN immunization combines most of the advantages of mucosal and i.m. immunization, in generating local mucosal, regional lymph node, and central splenic and circulating immunity. Furthermore, an alternative s.c. route of immunization achieves mucosal immunity with two injections, as compared with five mucosal applications; this reduces the experimental time and expense by more than 50%. This route of immunization may facilitate investigations of potential vaccines against HIV and other sexually transmitted diseases because broadly based and consistent mucosal immune responses are induced. The adjuvant used is aluminium hydroxide, which has a long safety record in humans. TLN immunization may also facilitate compliance and allow flexibility in administration of a

vaccine in different countries, because s.c. immunization might be more acceptable to some than genital or rectal immunization. Other sexually transmitted diseases that are open to this approach are human Papillomavirus, herpes simplex, *Candida*, *Chlamydia*, or gonococcal infection. The functional significance of the four levels of immunity will need to be tested by mucosal challenge with SIV or other microbial agents.

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